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16. Abstract

First the authors review centrifuging and clinostat experiments designed to determine the effect of gravity on the development of embryos. They then discussed their own experiments. They conclude that although it appears that the effect of zero gravity on development is negligible, further studies must be done.

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TABLE OF CONTENTS

IV.1 On the Physical Mechanism of the Effect of Gravity on the Embryo..... 2

IV.2 On the Effect of Gravity on the Early Development of Amphibians..... 7

.1 The First Experiments on Changes in the Orientation of Eggs in the Gravitational Field..... 9

.2 Experiments Involving Moderate Centrifuging of Eggs..... 14

.3 Further Experiments Involving Revolutions of the Eggs... 19

.4 Clinostat Rotations as a Means of Imitating Zero Gravity..... 28

.5 On the Zonal Organization of the Fertilized Amphibian Egg..... 33

.6 The Effect of Clinostat Rotation on the Process of Ooplasmic Segregation..... 37

.7 The Effect of Clinostat Rotation on Gastrulation..... 45

IV.3 The Effect of Gravity on the Development of Other Vertebrates..... 50

Figures..... 55

References..... 61

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ROLE OF GRAVITY IN EARLY DEVELOPMENT

The special question of the effect of gravity on development /140* was one of the first problems in experimental embryology and since the time that it was originally discussed it has been considered in the light of conflicting approaches to the fundamental problems of morphogenesis. Actually, the question concerns the interpretation of experiments which involved rotations and moderate centrifugal and clinostatic rotations of developing eggs, experiments around which stormy discussions have unfolded over the course of many years with the object of determining the relation of external factors to the development of the embryo. We list the individual questions which have been considered: on the role of the regular distribution of the various components of the egg, on the nature of the polar and bilateral organization of the embryo, on the relation between the cortical layer of the egg, its nucleus, and its cytoplasm, and, in general, on the subsequent modification in the spatial structure of the embryo. It would be very bold to assert that a single one of these problems will be completely resolved at the present time.

Recently the question of the effect of gravity has unexpectedly acquired practical importance in regard to the beginning of the space age. Until now direct investigations into the effect of

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zero gravity on embryogenesis have encountered significant technical difficulties and we must therefore rely basically on data which were obtained by the classical methods of experimental embryology.

In addition, we must specify one special circumstance: the bulk of these data were obtained from experiments on amphibian embryos while the data on the effect of gravity on the development of other vertebrate species are very scanty and no work has begun to study this problem as applied to invertebrates.

IV.1 On the Physical Mechanism of the Effect of Gravity on the Embryo

Before we turn to a discussion of the primary mechanisms involved in the effect of gravity on development we recall several well known physical principles.

Sufficiently small elements of two bodies which have random forms and dimensions may be considered material points, the masses of which are equal to the compositions of their volumes (dV_1 and dV_2) on their densities (ρ_1 and ρ_2). Between any two material points there are forces of mutual attraction which are directly proportional to the compositions of the mass of these points and /141
inversely proportional to the square of the distance between them $\bar{F}_{12} = \gamma m_1 m_2 / \bar{R}^2 \cdot \bar{R}_{12}/R^3$, where F_{12} is the force of gravity acting

on a point with mass m_1, $\overline{R}_{12}$ is the radius vector which goes from this point to a point with mass m_2, $R=|\overline{R}_{12}|$ is the distance between the points. The coefficient γ is called the gravitational constant (the constant of gravitation). Its numerical value depends only on the units of measurement selected:

$$\gamma=(6{,}67\pm0{,}01)\cdot10^{-11}\,\text{nm}^2/\text{kg}^2=(6{,}67\pm0{,}01)\,10^{-8}\ \text{dyne}\cdot\text{cm}^2/\text{g}^2.$$

The force of gravity $d\overline{F}_{12}$ acting on an element of one body from all of the elements of the other body is equal to $dF_{12}=\gamma\rho_1 dV_1\int_{V_2}\frac{\rho}{r_{12}^3}\overline{r}_{12}dV_2$,

where integration is done with respect to the entire volume V_2 of the second body.

Since in the first approximation the earth is spherical and its mass is distributed according to spherical symmetry, the force of gravity towards it in a fairly small body of random form and mass m_1 equals $\overline{F}_{12}=\gamma m_1 m_2/R^3/\overline{R}_{12}$, where m_2 is the mass of the earth, $\overline{R}_{12}$ is the radius vector linking the body with the center of the earth, and $R=|\overline{R}_{12}|$. We recall that a force $\overline{P}$ which a body which is immobile with respect to the earth exerts due to its attraction to the earth is called the mass of the body. The weight of the body is equal to the vector difference between the force $\overline{F}$ of the attraction of the body to the earth and the centripetal force $\overline{F}_c$ which determines the participation of the body in the diurnal rotation of the earth $\overline{P}=\overline{F}-\overline{F}_c$, while $F_c=m\omega^2R\cos\phi$, where

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m is the mass of the body, ω is the angular velocity of the diurnal rotation of the earth, R is the radius of the earth, and ϕ is the latitude of the observation point.

The movement of the body under the influence of a unit force equal to its mass is referred to as free fall. Free fall acceleration is the same for all bodies and like their masses, depends on their latitude and elevation above sea level. The numerical values g (cm/sec^2) for free fall acceleration at low elevations above sea level may be determined from the approximate formula $g=978,049\ (1+0.005288\ \sin^2\phi-0.000006\ \sin^2 2\phi)-0.0003086h$ where h(m) is elevation. At the geographic poles ($\phi=90°$) $F_c=0$ and the mass of the body is equal to its force of attraction to the earth. Consequently, the radius of the earth and centripetal force depend on latitude, the mass of the body is maximum at the poles and minimum at the equator. However, the difference does not exceed 0.55%. As concerns the question of the effect of gravity on development, these differences may undoubtedly be disregarded.

The standard normal value of g which is used for barometric calculations and for constructing a systems of units equals 980.665 $cm/sec^2 \approx 981\ cm/sec^2$. Thus, all life on earth exists under the influence of a constant gravitational force at a constant magnitude.

Practically speaking, what is the fundamental effect of gra-

vity on development from a physical point of view? The view has been repeatedly expressed that gravity often causes unequal mechanical stresses to arise in tissue and that these stresses may affect growth (Pollard, 1965). This is true for the postnatal development of connecting tissue, in particular, muscle tissue. It is already known that the growth of the majority of bones is to a significant degree controlled by stresses to which they are subjected as they fulfil their support functions.(Jager, 1870a, b; /142 Nordin, 1970; Smith, 1975). We imagine that when such stresses arise in the cortical layer of an egg or in embryonic tissue they may affect formation, this is especially so since, as was shown, the structure of mechanical stresses is actually linked to the morphogenetic movement of the cells (Weiss, 1929; Beloussov et al., 1974, 1976a; Beloussov et al., 1975). However, it is evident that in virtue of the small dimensions of the egg, and furthermore of the fact that it is usually immersed in a liquid of density similar to its own, these stresses are incommensurably less than those which arise due to changes in the form of the cells during morphogenesis. It is furthermore well known that the dimensions of the eggs vary considerably within a single clutch, and that since the stresses which arise due to gravity in the liquid filled membrane are directly proportional to the square of the membrane's linear dimensions, they vary significantly from egg to egg. Consequently, the proposed dependence of morphogenesis on these stresses is, practically speaking, incompatible with the regularity of morphogenetic reproducibility in the population.

It is not worth while to discuss the possibility of the direct interference of gravity in the biochemical process. The energy which is expended and released in individual biochemical reactions is several orders of magnitude greater than the work required to transpose molecules which participate in reactions in the gravitational field.

Since we cannot seriously consider speculations on the presence of specialized "gravity receptors" in all animal and plant cells (for detailed discussions see Pollard, 1965, 1971; Salisbury, 1969), only a single reasonable possibility remains: if gravity in general plays a role in development, then its effect is essentially that of maintaining the regular spatial distribution of cytoplasmic components which have different densities.

However, in this case we must consider fairly strict physical limitations. Due to thermal motion, the Boltzmann concentration distribution for each component of the cytoplasm with a given density is formulated on the basis of height: $\sigma(h) = \sigma(s)e^{\frac{-V(\rho-\rho_0)gh}{kT}}$ where $\sigma(h)$ is its concentration at height h, ρ is the density of the cytoplasmic inclusion, ρ_s is the density of the cytoplasm surrounding it, g is the value of the free fall acceleration, ν is volume, $k=1.38 \times 10^{-16}$ erg/degree is Boltzmann's constant, and T is absolute temperature. It is simple to determine that for cytoplasmic inclusions of dimensions less than 0.05 μm, the difference between the concentrations in the upper and lower parts of

the still undivided egg (not considering cells of smaller dimensions) will be negligible. Thus, gravity by itself does not affect the distribution of even relatively large macromolecular complexes.

On the other hand, it also follows from this that gravity must affect the distribution of such large cell organs as the yolk granules which occupy a considerable portion of the eggs of most animals.

The first to turn his attention to this was Jager in 1870 (Jager, 1870b). He was the first to propose that the animal-vegetable polarity in the eggs of frogs is related to the distribution of the yolk due to the effect of gravity.

IV.2 On the Effect of Gravity on the Early Development of Amphibians /143

We must first explain why this question has at various times been considered of primary importance to experimental embryology. It is true that the animal-vegetative polarity in the eggs of amphibians is primarily evident in the unequal distribution of components of the yolk according to their specific gravity: larger, densely packed yolk granules (or "yolk membranes" as they are called) are found in the vegetative hemisphere while fine yolk granules are found in the animal hemisphere. After fertiliza-

tion and the formation of a transvitellin area, the egg rotates continuously so that its vegetative hemisphere is below and its animal hemisphere above. Since segmentation is complete, cells participate in morphogenetic movements; these cells contain the most varied yolk fractions, from the lightest to the heaviest. It turns out that the behavior of these cells is varied in many respects, from differences in the rate of segmentation to very fine differences in the character of morphogenetic movements. It is visually apparent that differences in the behavior of spatially divided cellular populations are directly related to regularities in the spatial distribution of components of the egg at the earliest stage of development.

Until now it has been considered reasonable that the spatial structure of the embryo must be determined either from differences among sections of it during early stages of development, or from the spatial irregularity of the external conditions in which different stages of morphogenesis of the entire embryo, or of its components, occurs (Gustafson, Wolpert, 1961; Wolpert, 1972). For the majority of factors external to the embryo, their participation in morphogenesis is related to the fact that they are highly variable and act isotropically on the embryo. This question must be considered experimentally as concerns gravity.

This experiment was naturally constructed on the basis of changes in the orientation of the egg in the gravitational field

since it was impossible to eliminate the effect of the earth's gravitation. As the classical "development mechanism" proposes, embryogenesis is an unambiguous link relating cause and effect. It has always been assumed that in this type of experiment the development of the embryo is either completely normal or completely anomalous and that the effect of gravity is evident in the mechanisms of development. It will be obvious from the nature of the anomalies whether or not this occurs.

IV.2.1. The First Experiments on Changes in the Orientation of Eggs in the Gravitational Field

The first such experiment was done by Pfluger in 1883 (Pfluger, 1884; see Bogdanov, 1888). Pfluger was able to prevent the rotation of the egg, which begins after fertilization, in the yolk envelope by placing the unfertilized egg on a dry glass sheet and fertilizing it in a small drop of water. Due to the small amount of surrounding liquid after fertilization, a full-fledged trans- /144
vitellin space which is wide enough to allow the egg to rotate in the envelope and to orient itself down from the vegetative pole does not arise after the removal of the yolk envelope. Thus, after the glass sheet with the egg adhering to it is inverted, the egg remains oriented upwards of the vegetative pole. It is clear that numerous cases of anomalous development are difficult to interpret because many of the conditions under which the sura usually develops change simultaneously. Pfluger

emphasized that in separate instances normally constructed larvae were hatched from these eggs, however, there is practically no pigmentation in the head region which is usually developed from the animal hemisphere of the egg. At the same time Pfluger noted that the first segmentation sulci in the inverted eggs were not at the animal, but the vegetative pole. This was true despite the fact that the pigment was not transferred from the animal to the vegetative pole. From this, Pfluger drew the conclusion that gravity has a direct effect on the orientation of the first segmentation sulci and on the position of the basic axes of the embryo while the distribution of the components of the egg by itself has no such effect.

This conclusion was immediately subjected to severe criticism (Roux, 1884; Hertwig, 1885) and within a year Born had revealed from his study of histological preparations of such eggs that although the peripheral layer in the animal section in which the pigment is concentrated does not actually shift after rotation, the entire internal cytoplasm is redistributed in such a way that the heavy yolk runs along one of the walls of the egg to a new equilibrium position and the cytoplasm of animal origin is forced up into the region of the formerly vegetative pole (Born, 1884, 1885a, b). The nucleus is shifted to this region and is there formed axially while the sulcus of the first segment is cut opposite to it. Born made another and subsequently pivotal observation that the wall along which the yolk runs partially loses its

pigmentation and a grey falx is found there. This is referred to as the artificial Born grey falx (Pasteels, 1964). It was shown later that the position of the artificial grey falx in these embryos determines the position of the dorsal lip of the blastopore and, subsequently, the density of bilateral symmetry (see Clavert, 1962).

Thus, despite the conclusion drawn by Pfluger, it is clear from his own experiements that if the force of gravity affects the distribution of the basic axes of the embryo, it is only by means of redistributing the internal contents of the egg.

It is true that these experiments did not deal at length with the role of gravity in normal development. We recall that even in oogenesis, the egg has an animal-vegetative polarity irrespective of gravity, while not yet laid eggs have random orientations of their poles relative to the vertical. It appears incomprehensible that gravity might supplement this polar orientation.

Thus, in Roux's opinion, it is not a "necessary factor in development" (Roux, 1887, 1895). However, what is such a "necessary factor"? There is no a priori basis for asserting that a single factor is responsible for the formation of polarity in /145
oogenesis and at the same time the reason for maintaining oogenesis in the last stages of development..

This construction suggests the possibility of rotating the egg not at the moment of fertilization, but somewhat later. This was first done by Schultze from the stages of two, four, and eight blastomeres in a frog embryo (Schultze, 1894a,b, 1900; Wetzel, 1895). Pfluger's method was clearly not suitable for these experiments. In order to prevent the egg from returning to its original orientation in the gravitational field after it had been rotated, Schultze compressed it between two parallel glass sheets. The gap between the sheets was calibrated in advance so that they would not squeeze the egg too tightly (which is known to cause anomalous fragmentation by itself), and at the same time the the yolk envelope was not perforated. In all cases fragmentation was somewhat anomalous; divergences from the development of the control embryos, which occurred between similar sheets but in a normal orientation, were particularly impressive when the experiment was begun from the two blastomere stage. In several cases the embryos exhibited distinct twin deformations. Schultz himself interpreted this result as following from a partial disturbance in the first two blastomeres which developed as if they had been isolated from each other. As we will demonstrate below, this interpretation was completely inaccurate.

It is especially important to emphasize that some of the embryos in Schultze's experiments developed more or less normally, and that the relative proportion of eggs which developed anomalously depended on the angle of inclination of the

animal-vegetative axis of the egg towards the vertical and on the temperature at which the experiments were conducted. For example, the lower the temperature, the more frequent were twin deformations.

This essentially allowed Roux to present the following, remarkable point of view. Roux asserts that gravity does not exert an organizaing effect on formation and that the spatial organization of the embryo is determined once and for all by a given and unchanging sequence of internal factors. External factors may only distort the development mechanism; the fact that inverted eggs develop anomalously only indicates that they exist in anomalous conditions and does not permit us to conclude, as did Schultze, that gravity is related to the normal development mechanism or that it is, moreover, a necessary factor (Roux, 1897; 1900a,b; 1902; 1903). Thus, the variable sensitivity of eggs to gravitation is related simply to differences in the "stability" of some internal mechanism. Unimpaired parts of the mechanism continue to work: for example, the position of the plane of bilateral symmetry after rotation which follows fertilization depends on the position of the artificial Born grey falx
and not on the point of penetration of the spermatozoon. Roux explains it by the distortion of the course of the spermatozoon in the egg, the structure of which changes due to the overflowing of the yolk. At the same time, Roux proposed that before it fuses the pronucleus lies in a plane which coincides with the future sagit-

tal plane of the body, i.e., that the bilateral organization of the embryo is determined only by the internal mechanism which acts in normal development and that this organization remains intact. In /146 any case this proof is too categorical and in some circumstances is simply incorrect.

IV.2.2 Experiments Involving Moderate Centrifuging of Eggs

Roux's idea of the inalterability of fully determined internal factors in the spatial arrangement in the egg is specifically contradicted by results of experiments which involved moderate centrifuging. In these experiments, segmentation proceeded under the influence of factors external to the embryo.

Beginning in 1899 and continuing to the recent past, many experiments were done which involved centrifuging amphibian (primarily ecaudate))eggs, however, most of the basic results were obtained by Oskar Hertwig who did the first experiments (Hertwig, 1899). He centrifuged the egg of Rana esculenta and Rana fusca at acceleration 1.5-2.5 g beginning 3 hours after fertilization and continuing until the end of segmentation. Evidently acceleration was very low but the exposure time was very long. Even at the lowest acceleration (1.5 g), irregularities in segmentation noticeably increase, so that by the end of segmentation the differences between the animal and vegetative blastomere sizes has become much larger than usual. These differences are regu-

lated however, and the embryo proceeds to develop normally. At acceleration on the order of 2.5 g, after 1 hour of exposure, the segmentation of the vegetative hemisphere of the egg is overwhelmed and after 24 hours the animal hemisphere occupies a porous mass of finely comminuted cells which lies in the integral yolk. Finally, segmentation is also meroblastic when centrifuging is done at intermediate accelerations; pigment from the animal hemisphere runs down to the equator, however, the disintegrated cells form a well ordered disk, or "blastodisc", the end of which is subsequently turned under, as if in an attempt at gastrulation.

We may note several more or less constant features of meroblastically developed eggs. First of all, not only the animal-vegetative differences in the character of segmentation become very pronounced, but segmentation loses its order even locally--very large cells may be brought into contact with very small cells although they are not mixed together to form a single cellular disk. Numerous sulci in the vegetative hemisphere are smoothed after a fairly long time and the cytoplasm acquires the structure of a syncytium.

Finally, it is worth noting that even when even when there is only a slight irregularity in segmentation, the movement of the cells during gastrulation differs from normal movement. However, in such cases the embryos continue to develop and eventually acquire a completely normal structure.

It is evident from this experiment by Hertwig that a factor external to the embryo determines the degree of irregularity of segmentation: the greater the acceleration acting on the egg, the more pronounced the irregularity. In particular, the inverted egg experiments which were done by Hertwig and his student Wetzel have confirmed this (Hertwig, 1904; Wetzel, 1904). In these ex- /147
periments, eggs of the same species of ecaudate amphibians were centrifuged, however, prior to centrifuging, the eggs were fixed by Pfluger's method so that their vegetative poles pointed in the direction of the axis of rotation. The eggs segmented in a distinctly irregular manner, however, at this time, the cells from the vegetative hemispheres appeared to be finely segmented.

Hertwig noted that if centrifuging was begun immediately after fertilization, development was more anomalous. However, it is clear that in experiments which involved lengthy and very weak centrifuging, it was not easy to determine the basic stages of development which were particularly sensitive to the effect of centrifuging. This was done somewhat later (Morgan, 1902a, 1906; McClendon, 1909, 1910; Jenkinson, 1915; Banta, Gortner, 1915; Kas'yanov, Popov, 1971). In these experiments, the eggs of various species of amphibians in various stages of development were subjected to centrifuging for several minutes at accelerations of 200-2000 g. Clearly this resulted, to a greater or lesser degree, in stratification of the contents of the egg according to density. From this point, development proceeded more or less anomalously.

It was proposed that in this way it would be possible to separate clearly distinct periods of high sensitivity to centrifuging from each other. Actually it turned out that all stages of development up to gastrulation were more or less subjected to centrifuging effects and that it was only with considerable difficulty that it was possible to separate three such periods in which sensitivity rose: the first was the moment of the penetration of the spermatozoön inside the egg, the second was directly before the third (equatorial) sulcus was cut (Konopačka, 1908), and the third corresponded to the stage of the late blástula.

Evidently, the periods in which sensitivity rises are essentially different from each other. The existence of the second period is undoubtedly related to the fact that just as centrifuging has been completed, a slow shift of displaced components in the egg to their original positions begins, with the result that after 10 minutes, the boundaries between layers of cytoplasm of various densities (cytoplasmic inclusion) are eroded (Gurwitch, 1904, 1909; McClendon 1909). Clearly, if at this time the third equatorial sulcus is cut, it splits off components which have not yet returned to their normal positions. Incidently, such ideas easily explain the general reduction in sensitivity to centrifuging in terms of segmentation.

As was demonstrated later (Pasteels, 1953, 1964), until the third period the roof of the blastocoel collapses during centri-

fuging. Until this time, the roof is fairly thin and presses the presumptive entoderm of the blastula which induces supplementary axial structures. Clearly, this is not directly related to the subject of the present article.

Even in his first experiments, Hertwig noted significant variations in the total sensitivity to centrifuging and in the specific character of anomalies. Both variations hold for a single clutch of eggs. However, as Hertwig himself emphasized, a stereotype shift to meroblastic development is maintained in the anomaly ; this shift is nonspecific for a given effect. It has become known recently that this shift may actually arise due to numerous and varied factors (see, for example, Whi- 7148
tington, Dixon, 1975).

It would seem that the variability which was observed by Hertwig is related to the fact that low acceleration centrifuging is weak and therefore redistributes the contents of the egg in a highly variable fashion and serves only to separate completely its components, with the result that the contents become not only sharper but more standardized (it stands to reason that we are not concerned with centrifuging which is strong enough to suppress development completely). It is naturally hoped that it will be possible to determine the morphogenetic role of various parts of the cytoplasm. The results of experiments which involved short term but powerful centrifuging and which separated the contents of the egg

into a series of relatively homogeneous zones did not justify this hope.(Brachet, 1972). On the contrary, the more completely homogenized a zone of the egg was, the more it loses its ability to begin the formation of an embryo, irrespective of which part of the cytoplasm contains it. In addition, the variability of the anomalies which arise after centrifuging increases sharply against the background of the suppression of development.

IV. 2.3. Further Experiments Involving Revolutions of the Egg:

Despite the fact that the general sensitivity of the embryo to rotations and centrifuging is maintained to one degree or another during the entire segmentation, we must isolate developmental phases before and after the formation of the grey crescent falx. The effect of gravity manifests itself in qualitatively different ways in these phases.

The future position of the plane of bilateral symmetry in the egg is not fixed until the appearance of the grey falx and may depend on factors external to the egg; this was demonstrated in particular by numerous experiments conducted by Ansel' and Vintemberzhe (see Clavert, 1962). It is well known that if the egg is normally oriented in the field of gravity, at least in 75% of the cases (Roux, 1903), the plane of bilateral symmetry will pass through the point of penetration of the spermatozoon. However, if the egg is rotated,and allowed to revolve freely in

the yolk envelope, it will return to its original orientation, so that even such a brief stay in an inclined position may alter the position of the plane of bilateral symmetry relative to the point at which the spermatozoon penetrates. In this case, precisely as in experiments in which the slope of the animal-vegetative axis towards the vertical is fixed for a long time, the dorsal lip of the blastopore is on the side of the egg which is on top during a brief inclination.

If several of these short term inclinations succeed one another, the position of the plane of bilateral symmetry will take the direction of the last one. The greater the angle of rotation of the egg and the longer it stays in an inclined position, the more successfully its inclination competes in the determination of the plane of bilateral symmetry with the point at which the spermatozoon penetrates.

Related investigations of the internal structure of the egg have shown that the place at which either the artificial or natural grey falx will arise is evidently determined by the asymmetry of the form of that internal region of the egg which is filled with heavy large yolk granules (Roux' "white vegetative yolk"). Characteristically, the grey falx appears where the heavy yolk is closest to the cortex. /149

Thus, before the appearance of the grey falx,

the egg has an infinte axis of symmetry and an infinite number of planes of symmetry crossing it: after the grey falx has appeared only one such plane remains. Due to a clear misunderstanding, the transfer of the egg to bilateral organization is termed "symmetrization", while the characteristic transposition of the cortical layer which is observed when the grey falx appears is termed "rotational symmetry" (see Clavert, 1962). In fact, it is not difficult to see that the egg loses rather than acquires elements of its symmetry, so that what actually occurs is asymmetrization (Shubnikov, Koptsik, 1972).

Considering that no series of physical phenomena occurring within a system can decrease the order of symmetry in the system (the Curie principle), we are compelled to believe that the transfer of the egg to bilateral organization is impossible without the directed interference by external agents (Kiebel, 1902; Moszkowski, 1902). There must be "sufficient bases" for the egg to select one of the initially equivalent planes of symmetry. Nevertheless, the system must arrive at the situation in which its symmetrical spatial configuration is unstable, and then, with as slight a disturbance as necessary, it rapidly transfers to a new, stable configuration. When this happens, naturally some elements of its symmetry are lost.

We must consider that precisly in such situations we will encounter a grey falx. Actually, a complete-

ly defined and reproducible result may arise due to various and sufficiently weak interactions: not only the penetration of the spermatozoon and brief changes in the orientation of the egg in the gravitational field, butaalso its light compression when placed in a weak temperature gradient or an aeration gradient. (Penners, 1936; Løvtrup, 1965).

In this case, all of our interest must be turned to the question of at what level of organization and in what manner the system becomes unstable, rather than to the nature of random disturbances which arise in the system due to this situation. It stands to reason that this is related to the effect of gravity on the establishment of bilateral organization in the embryo.

Assume, however, that polar and bilateral organization have already been established. The question remains of the mechanism which maintains continued development. We know that in Xenopus laevis, fragments of the cortical layer from the grey falx region may induce additional axial structures until the stage of eight blastomeres if they are transplanted to the ventral side of the egg which exists at every stage from the moment of fertilization to the cutting of the third (equatorial) sulcus (Curtis, 1960). At these stages, the removal of the cortical layer from the grey falx region suppresses the development of a fundamental axial complex in the embryo. We may conclude that dorsoventral discrepancies are fixed in the cortical layer of the egg

for cutting the equatorial sulcus. Then, however, these discrepancies are maintained at the level of the internal structure of the presumptive entoderm--its internal polarization. This question was studied in great detail in a special survey by Nieuwkoop, (Nieuwkoop, 1973).

The significance of the internal structure of the egg in /150
maintaining the bilateral organization of the embryo is emphasized by the fact that perhaps the most crucial and more or less regularly reproducible result of experiments involving rotations of amphibian eggs after the grey falx emerges is the appearance of various types of twin deformations, i.e., of duplications in the axial structures. This was investigated in a highly detailed survey by Penners and Schleip (Penners, Schleip, 1928a,b; Penners, 1929, 1936). Primarily they demonstrated that such duplications do not arise . . . due to the dissociation of development in the first two blastomeres, as proposed by Schultze (see also Morgan, 1895).

It appears that the character of the anomalies which arise is critically dependent on the manner in which the yolk flows after rotation. The heavy yolk, which has been shifted to a new equilibrium position, basically flows along the internal surface of the zone of contact between the blastomeres. A shift in the blastomeres filled with heavy vegetative yolk in the direction of the former animal pole was observed in the rotated egg until the blas-

tula stage. However, characteristically twin deformations are regularly reproduced only when the experiment begins before the equatorial sulcus is cut; the earlier the experiment is begun and the lower the temperature, the more frequently these deformations are reproduced (obviously, this is within physiologically allowable limits). Additional blastopore lips are located where cells which contain heavy vegetative yolk, on the surface of the embryo or close to it, are in contact with cells which contain cytoplasm of animal origin. Gastrulation begins in several spots on the boundary between dark and light spots (or poles) formed on the surface of the embryo; however, this does not occur simultaneously. Additional regions of the primary organizer compete with each other, flow together, and as a result, several types of more or less "regularly" constructed embryosswith multiple axial structures are formed. We note in this case that those parts of the additional lip which are in proximity to the original dorsal side of the egg dominate (i.e., they appear first and are developed in preference to the others)-(Pasteels, 1938, 1939).

The basic results obtained by Penners and Schleip in their experiments on "the Schultz rotation" were recently confirmed in similar experiments (Brachet, 1972; Svyatogor, Svyatogor, 1974). After Nieuwkoop, these and similar experiments were interpreted in the light of the induction of additional mesodermal structures by a presumptive entoderm. However, if the role of the inductor, in precisely the same way as the role of a reacting tissue, is fixed

from the beginning at cells of defined origin (i.e., which contain completely determined cytoplasmic components of the egg), then it is difficult to understand how a single region of the embryo is able to fulfil two roles: that of inductor, in relation to one of the neighboring regions, and that of reacting tissue, in relation to another. Pasteels noted (Pasteels, 1939) that if the egg is not released from the pressure of constricting plates during rotation experiments, then involution of the material through the blastopore lip which is located on the boundary between "black" (animal) cells and "grey" cells which contain vegetative yolk is impossible for purely mechanical reasons. Somewhat later, attempts at gastrulation may be observed at less sharp boundaries (for example, on the boundary between "grey" cells and practically "white" cells). Since these attempts are unable to continue for the same reason, attempts at gastrulation proceed at decreasingly sharp boundaries. If we consider that a given region, for example, one which contains grey cells, is capable of the induction of a blastopore lip, and at the same time is able to respond to this inductor, it is unclear why the blastopore lip is not located in this region, but on its boundary with other regions. /151

It is more natural to think that additional organizers arise very rapidly in more heterogeneous regions of the egg (i.e., in the vicinity of boundaries between regions which are sharply distinguished from each other according to the properties of their

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components). The origin of the material and the part of the cytoplasm which composes it is essentially unimportant; its degree of heterogeneity is crucial because the greater it is, the more rapidly the material will be divided during the next stage of epigenesis into sections with different potentials.

Thus, as we have noted previously, after the formation of the grey falx, the determined order in the distribution of the internal components of the egg is especially significant to maintaining polar and bilateral organization, it is all the same whether the question is of the spatial distribution of some "determinants" (Flickinger, 1970; Kosher, 1973; Melton, Smorril, 1974; Malacinski et al., 1975; Bruce et al., 1975) or of the mutual arrangement of various heterogeneities within the egg. It is evident from the extreme sensitivity of this distribution both to changes in the egg's orientation in the gravitational field and to even low acceleration centrifuging, that gravity must affect the egg when it develops under "normal conditions". We note further that sensitivity to rotations is maintained even at the latest stages of development (until the beginning of gastrulation), although twin deformations do not occur with such regularity and individual variability is even more pronounced.

From this entire series of experiments it follows that despite the absolutely clear and unchanging physical nature of the stimulus, the response to it is always extremely variable.

Thus, in these experiments, individual differences in the structure of the eggs which are never found in normal development were unexpectedly discovered.

The reasons for this are easy to understand if weerecall one inherent difference between gravity and the majority of other factors in the region surrounding the embryo. Actually, gravity always, and in this case, unalterably affects the range of evolution, and consequently, selection may not assign mechanisms which guarantee resistance to changes in this parameter of the external atmosphere. If, for the normal development of the egg, it is necessary to stabilize its polarity, and this polarity corresponds (it may be maintained) to the distribution of components of various densities, then if a mechanism is constructed for maintaining the standard position of the egg in the gravitational field, the necessity for other mechanisms which stabilize polarity is considerably lessened. In other words, if a stable means of developing polarity has already been formed ("a creode" according to Waddington--Waddington, 1939), then for those parameters, variations in which do not lead the system to the limits of the creode" the stabilizing selection pressure is removed (i.e., the selection in genotypes which stabilize the values of these parameters is absent). /152

Thus, when gravity is eliminated, we must expect a destabilization of the creode: it is natural that the response to this

stimulus will be highly variable within a population and must be strongly dependent on numerous transient circumstances in development which usually exert only slight influences on the outcome of development. For example, qualitative results from rotation and centrifuging experiments depend strongly on temperature.

IV.2.4. Clinostat Rotation as a Means of Imititating Zero Gravity

Clinostat rotation is the slow and regular rotation of an object around a strict horizontal axis. If the time of one rotation of the clinostat is much less than the characteristic time necessary for an essential change in the spatial configuration to occur in the system under study due to the effect of gravity, then all of the components of the system take the positions which they would occupy in zero gravity shortly after clinostat rotation. (or, more precisely, they oscillate with a minimum amplitude near around these positions). However, in this case, the rotation velocity of the clinostat must be low enough to allow us to disregard centripetal forces which arise, i.e., the following relation must hold: $\omega^2 R \ll g$, where R is the maximum radius, ω is the angular velocity of rotation of the clinostat, $g = 9.81$ m/sec^2 is the free fall acceleration.

The group of biological objects which satisfy both of these conditions is small. Nevertheless, due to their small sizes and relatively slow reactions to changes in their orientations in the

gravitational field, clinostat rotation may be an adequate means of imitating zero gravity for developing eggs of many animals, at least until fast-reacting sensory systems have developed.[1]

Following Sachs (Sachs, 1882: see Pfeffer, 1904; Zarsen, 1962; Gordon, Shen-Miller, 1971), who used clinostat rotation to investigate geotropism in plants, Roux (Roux, 1884) attempted to answer the question of whether gravity is necessary for normal development experimentally. He placed the eggs of a grass frog on a water wheel rotating slowly around a horizontal axis. In another variant of the experiment, the eggs were placed in a long test tube which was oriented along the radius of the wheel rotating in the same way. During each rotation, the eggs twice slid from one end of the test tube to the other and changed their orientation in the gravitational field. This experimental scheme was subsequently used by Morgan (Morgan, 1904) who, in order to increase the effect of reorienting the eggs, embedded them in a glass spiral test tube. In other experiments, Morgan had the eggs develop in water which was continuously agitated by air bubbles (one complete rotation of the clutch took 5-15 sec.) (Morgan, 1902b). Finally, Kathariner had a clutch rotate around /153
a horizontal axis which was lowered through a vessel in which there was a strong water current (Kathariner, 1901; 1902). We

1. In this survey we do not consider data on the direct influence of zero gravity on the formation of sensory systems and other organogeneses (see Pal'mbakh, L. R., 1976a,b; Baumgarten et al., 1975).

must emphasize that in all of these experiments the question was raised as to whether gravity is a "principally necessary" factor in development, i.e., a result is considered negative if the development of part of the egg is normal. Strictly speaking, this result was obtained. Clearly, the number of experiments was limited and anomalies which arose during the early stages of development and were subsequently corrected could easily have escaped the attention of the investigators.

However, it was observed in one clinostat experiment that anomalies of development occurred in a part of the embryo of Xenopus laevis and Rana pipiens as a result of the stimulus. Unfortunately, this experiment did not trace the course of development and the result shows the situation after five days of clinostat rotation. Furthermore, at rotational velocities (1/4-2 rev/min), the eggs succeeded essentially in turning in their envelopes during the course of a single rotation. It is thus unclear how closely the results of these experiments are related to the imitation of zero gravity (Tremor, Souza, 1972; see also Young et al., 1970).

For several years the authors of the present article have studied how clinostat rotation, in imitating zero gravity, affects the early development of ecaudate amphibians (Dorfman, 1974; Dorfman, Cherdantzev, 1977a,b). In our experiments we used various modifications of a clinostat with a working chamber of diameter

30 mm. We worked with embryos of Rana temporaria, Rana arvalis, Rana esculenta, and Xenopus laevis. The eggs were rotated in the clinostat at velocities from 12-35 rev/min for 0.5-48 hr. beginning from various stages of early development, from fertilization to the neurula stage. Even at the lowest rotational velocity (12 rev/min), the time of one rotation of the clinostat was considerably less than the time in which the egg, if it was inverted, returned to its usual orientation in the gravitational field. In addition to the standard laboratory control, experiments in which the embryos were rotated about an axis at an angle of 45° to the vertical were run parallel to the main experiments. In these experiments development proceeded exactly the same as under standard laboratory conditions. The presence of a constant gravitational pull along the axis of rotation appeared to be a sufficient condition for development to proceed completely normally.

First of all we are struck by the fact that differences occur in the sensitivity to clinostat rotation even among eggs from the same clutch: some of the embryos develop practically normally while others are distinguished by the nature or by the degree of expression of anomalies. In precisely the same way, the embryos' abilities to correct damage that occurred in them differed; in completely similar cases of changes in morphology some embryos died, while development in others was corrected sooner or later. Even more pronounced were differences in sensitivity and ability

to correct damage from one clutch to another.

In the data from a single clutch we never see the entire spectrum of possible disturbances in development. An idea of the range can only be gained from a comparison of the anomalies which are regularly encountered in different clutches. We repeated a single experiment until we no longer observed qualitatively new anomalies. We may conclude from the diverse anomalies that their range is wider and degree of expression more pronounced the earlier clinostat rotation is begun and the longer /154
it is continued. The greatest sensitivity to the stimulus was observed in cases in which clinostat rotation was begun immediately after fertilization. If the embryos were taken in middle gastrula stages and later, no effect on development was noted.

The spectrum of anomalies which corresponds to a stage of development does not depend on whether clinostat rotation was continued until this stage or terminated long before. It is also regularly independent of the velocity at which the developing egg is clinostatically rotated. This fact, together with the absence of an effect from rotating the clinostat slope, indicates that oscillations of the components of the egg around an equilibrium position were so small that by themselves they did not affect development, and that consequently, in the selected range of velocities, clinostat rotation adequately imitates zero gravity.

IV. 2.5. On the Zonal Organization of the Fertilized Amphibian Egg

By comparing the size of the yolk granules which are distributed in the amphibian egg it is possible to discover that after fertilization, only yolk granules which are similar in size are arranged close to each other, i.e., that the yolk has a local order. Nevertheless, at opposite poles of the egg, granules are found whose dimensions differ by 2-2.5 times. The size of the granules changes continuously from one section of the egg to another. We have not yet discussed the boundaries across which the mean size of the granules changes spasmodically. Fig. 26 illustrates the zonal organization of the yolk in the grass frog egg. The boundaries run along those sections of the egg in which the local regularity of the yolk is disturbed. In each such section the yolk granules which correspond in dimension (maximum diameter) to the granules which fill the two sections of the locally homogeneous zones adjoining the given segment of the boundary are intermixed. The mean size of the yolk granules in such homogeneous sections which are in contact with the boundary may be reliably distinguished (the reliability of the difference in the mean sizes, 0.99 according to Student, is obtained from a sample of 10-15 granules from each section).

Thus, the egg is subdivided into regions which are locally homogeneous throughout their areas and which are separated by nar-

row transitional zones or boundaries in which granules from various regions are mixed (Fig. 26 A, B). This alone is completely sufficient to show that surface tension exists on the boundaries which divide regions containing yolk granules of various sizes. This surface tension is physically very complex and has, for example, nothing in common with that arising at an oil-water interface. It is rather, a direct consequence of the fact that the penetration of foreign granules in a neighboring region is, for some reasons, highly unfavorable, and that consequently, large transitional zones are highly unfavorable.

The equilibrium configuration of the system corresponds to the minimum potential energy E in which $E_0 = 1/2 \sum_{ij} \sigma_{ij} S_{ij}$ appears /155 as the free energy of the surface of the interface, where S_{ij} is the surface area of the interface of the i and j phases and σ_{ij} is surface tension, while potential energy is $E_g = g \sum_i h_i \rho_i V_i$, где h_i where h_i is the position of the center of gravity of the i phase, V is its volume, and ρ_i is its density. $E = E_0 + E_g$. All $\rho\rho$, V_i, σ_{ij} and g are parameters of E. In the absence of gravitation ($g = 0$), the potential energy of the system will be comprised exclusively from the free energies of the surface of the interface ($E = E_0$).

Let the system have a configuration which corresponds to the minimum potential E_0. In the gravitational field it slowly (due to viscosity) begins to take the configuration which corresponds to the minimum potential $E = E_0 + E_g$. During a rotation around the

horizontal axis, the direction of the effect of gravitation continually changes to its opposite and the configuration of the system will oscillate around the original one. In this situation, which corresponds to a minimum E_0, a clinostatically rotated system arises, regardless of the position of its components prior to rotation.

Inasmuch as the time in which the form of a zone of the yolk changes during rotation of the egg is several minutes, even at rotation velocities of several revolutions per minute, variations in the form of the regions of the egg will be negligible, i.e., the egg will always retain the structure that it has in zero gravity.

Eggs of a single clutch may differ considerably from one another in size as well as in the relative number, composition, and density of the individual components of the yolk. Despite this, in an important sense they all have essentially similar segregation structures. This similarity is related directly to the fact that gravity always arranges regions of the egg according to density in the same way, so that the light yolk is near the animal pole and the heavy yolk near the vegetative pole. Even when there are significant variations in the relative numbers of individual parts in the yolk and in the absolute values of the surface tensions on the phase interfaces, the characteristic elements of spatial order are preserved: namely, phases which contain

similarly sized yolk granules are, as a rule, near each other, and when we cross from zones in the animal hemisphere of the egg to those in the vegetative hemisphere, the size of the granules increases correspondingly.

This is well illustrated by Waddington and Thom's idea on the creode properties of development. (Waddington, 1964, 1964; Waddington, 1972; Thom, 1969, 1970, 1972). We may speak of the system as being in a creode of normal segregation in a fairly wide range of changes in the parameters of the potential $E \equiv E_0 + E_g$. However, in the absence of gravitation, when $g=0$, such variations of the parameters V_i and 0_{ij} in scale may lead to an abrupt change in the zonal structure of the egg, down to subdivisions in regions, fusion of some and division of others, of the phases of the yolk (Fig. 26, C, D).

In this way, the transition may be interpreted as a catastrophe, by Thom, i.e., as a qualitative change in the morphology of the process even if only one of the parameters (here, the value of g) exceeds the limit of the values for which the morphology of the given creode is structurally stable. The potential E_0, even with defined values of V_i and σ_{ij}, has, as a rule, several local minima, each of which corresponds to its configuration of the phases, and whose spatial structure is capable of changing catastrophically with small changes in σ_{ij} and V_i. Thus, variations in the internal stability of the egg which do /156 /157

not affect the normal course of development, in the absence of gravity, produce morphological variants.

During clinostat rotation we frequently observed that the boundaries of the grey falx became diffused and that it acquired an undefined, irregular form (see Fig. 27, A and B). Even when its form changed only slightly, the internal structure of the egg differed sharply from normal: separate phases of the yolk became more compact and, primarily, the animal-vegetative order of phases of different densities was abruptly disturbed. The structures of these eggs became much more variable than normal: after clinostat rotation, variations in spatial configuration which involved the proximity of phases with completely different densities and phases with similar sizes of yolk granules appearing in completely different sections of the egg became possible. At times the distribution of the phases may be extremely peculiar: a part of the heavy yolk may move far up, right up to the animal pole. In this case, we see its flow which forces back the pigment from the cortical layer externally (Fig. 27,B). /158

IV.2.6. The Effect of Clinostat Rotation on the Process of Ooplasmic Segregation

Now we demonstrate that clinostat rotation must inevitably affect not only the appearance of the equilibrium distribution of various components of the egg, but also the movement of the system from one spatial configuration to another (Klag, Ubbels,

1975).

It is well known that yolk granules of different sizes are regularly arranged between regions with different prospective values in the stage of the late blastula (Nakatsuji, 1975). At the same time we may ascertain that even in normal development the distribution of separate phases of the yolk during the early stages is highly variable. Evidently, what we see shortly after fertilization is related to the beginning of the spatial distribution and ordering of the components of the egg, i.e., to their segregation.

The segregation of the heterogeneous region of the ooplasma denotes its separation into "daughter" phases which are distinguished from one another according to the composition of the yolk (for amphibians, according to the mean size of the yolk granules) and which are more homogeneous than their "mother" region. Thus, the segregation of the initially heterogeneous region determines the divergence among the fates of its daughter regions and imparts individuality to the distribution of material in it. Evidently, the sequence of segregation may concentrate in a very narrow zone a component of the material which was primordially distributed throughout the egg in low concentrations.

At the same time, the origin of the cellular population which fills the region of the blastula with a single prospective value

and which contains yolk granules of relatively the same size, may not be traced to a determined region of the egg, even if, due to the variability in the distribution of the yolk it is directly prior to the beginning of segmentation. This population is a polyclone (F. Crick's term--Crick, Lawrance, 1975), i.e., for a long time it is supplemented by cells which originate from the adjacent regions of the segmented egg. It is clear that the yolk is successively supplemented by yolk granules of similar size which are mixed in from the surrounding yolk along with successive segregations. It stands to reason that the granules which, in virtue of their size, have to branch out to a polyclone, must, with the generation of the polyclone, be distributed in its surroundings. Inasmuch as all of the polyclones fill the adjacent regions of the blastula, we must demand that the yolk granules which are similar in size be arranged near each other for the phase to begin dividing. However, differences in the degree to which phases are separated from each other may, generally speaking, create an unordered mosaic in which phases which are sharply distinguished from each other by the sizes of their yolk granules lie in proximity and phases with similar granules are found far from each other. (Fig. 28, A). /159

We found this specifically during clinostat rotation. The development of such mosaic embryos proceeds anomalously, and the patchier the embryo, the more anomalous its development.

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Thus, we conclude that in normal development, segregation must be organized such that the degree of size similarity in the yolk granules corresponds to the degree of their spatial proximity. Howethen should we visualize the spatial order of segregation in normal development? The necessary order is maintained in spite of variations in the extent of division of the daughter phases, if between successive events in segregation they are able to occupy positions which correspond to their densities. However, it is clear from rotation experiments that such transpositions require several tens of minutes, i.e., much longer than the time it takes for the segregated structure to change perceptibly. Consequently, the process of dividing the mother phase into two daughter phases must itself be spatially ordered: the "heavy" daughter phase must be in contact with the "heaviest" phases of the surrounding medium from the beginning of its generation.

Now we turn to the characteristic division of an initially heterogeneous phase. We propose that in this phase an accumulation of similarly sized granules arises randomly; this accumulation is the embryo of a new phase. This embryo grows and attracts all of the new granules of the same strain, which collide randomly with it in thermal movement, to itself. However, this takes a considerable amount of time since the yolk granules are relatively large and form a very dense suspension in which their thermal mobility is severely limited.

The natural alternative is segregation which proceeds on the basis of a pre-existing concentration gradient in the granules of any of the divided strains. If segregation is very favorable, a macroscopic force will act on the granule in this gradient forcing it to move against the concentration gradient of granules of its size. Such a system is not resistant to spatial discontinuities in the distribution of granules of a given size and will attempt to subdivide into homogeneous phases.

Actually, in the picture of the zonal organization of the egg we see that some boundaries between phases are not closed: in the zone in which m i x i n g of the granules continues, a steep concentration gradient is established. This zone becomes continually narrower and eventually terminates in a slim boundary between phases which are separated from each other.

Clearly, the irregular distribution of granules of different sizes within the mother phase which arises due to gravity is sufficiently pronounced that the lightest elements in the daughter phase are situated above, in the region surrounding the light phases of the yolk and the heaviest elements move down into the region surrounding the heavy phases of the yolk. Then, despite significant variations in the sequence of division of the phases, there is a constant relation between the spatial proximity of the yolk granules and the similarity of their sizes (Fig. 28, B).

Thus, segregation which proceeds in the absence of gravitation actually leads to a mosaic of phases of different densities. /161
It is understood that gradients which determine the initial conditions for segregation do not arise only under the effect of gravity. With clinostat rotation we achieved not simply the surpassing of the limit of the creode of normal segregation, but its total disintegration. This may occur, for example, due to the fact that granules of intermediate size are accumulated on the boundary between two phases.

It is more or less evident that when opposing currents which mix the yolk granules with one another, yolk granules arise in the segregated phase, its form will change.

We observed that in the course of normal division the vegetative yolk regularly flows in the cortex towards a formed sulcus (Fig. 27, D). When formation of the sulcus is complete, the yolk flows back and is shifted to the sulcus of the succeeding fission. In clinostatically rotated eggs, the character of these shifts changes to a greater or lesser extent. The movement of the vegetative yolk no longer obstructs its gravity, and the yolk, which spreads along the formed meridional sulci, penetrates much further into the animal region. It is frequently unable to flow back completely since the sulcus of the succeeding fission has already been cut. In the late stages of division, this produces variations in the distribution of the vegetative yolk along the blastomeres of the end zone of the blastula (Fig. 27, E, F) (compare with data

of Grant and Young; Grant, Young, 1974).

It is fairly natural that, inasmuch as in the absence of gravitation, phases with yolk granules which are strongly distinguished by size come into contact with each other, the boundaries between them become considerably sharper (or, the transitional zones between such phases are very narrow). In fact, after clinostat rotation, a drastic constriction of the transitional zones and an increase in the stratification of the yolk occur. In addition, inasmuch as division sulci appear, as a rule, directly along the boundaries between the individual phases in such cases, it is clear that development surpasses the morphologies which lie near to the limits of the creode of normal division (see also Kubota, 1969; Whitington, Dixon, 1975; Dziadek, Dixon, 1975).

Thus, in the normal division of the frog at the stage of four blastomeres, small spiral displacements in the animal and vegetative poles occur. However, this does not lead to the appearance of a third sulcus from the equatorial plane, and in fact the elements of spirality rapidly disappear. If such shifts occur in clinostatically rotated eggs, then the third sulcus runs strictly along the boundaries between the animal and vegetative cytoplasm in each of the blastomeres and, because of this, experiences fissuring on the transverse of the meridional sulci (Fig. 27, G).

We now turn to one additional consequence of the increase in the stratification of the yolk during clinostat rotation. The division sulci, which run along the interface of the phases, separate blastomeres, each of which contains only one yolk fraction, i.e., division is much more irregular than normal (Fig. 27, H). Inasmuch as blastomeres with large amounts of heavy vegetative yolk divide more slowly than do animal blastomeres, the irregularity in division will continue to grow during clinostat rotation.

As a last resort, the first sulcus runs along the border /162
which separates the dome of the animal cytoplasm from the remaining mass of the yolk (Fig. 27, I). The thin layer of animal cytoplasm which is intercepted by this "paracortical" sulcus may be finely subdivided in the future when the division of the remaining portion of the egg is suppressed: as a whole, division does not have a meroblastic character which is typical of amphibians (Fig. 27, J).

It is remarkable, that in cases of an especially anomalous distribution of cytoplasmic components, the sulci echo local heterogeneities in the structure of the egg, acquire peculiar forms and are capable of branching (Fig. 27, K). They frequently remain uncompleted for a long time (Fig. 27, L) and are subsequently completely extended so that the contents of several of them flow together, or they are corrected. In the course of ad-

justment, however, sulci which are cut in various divisions may be joined together. If this effect begins early enough, many fertilized eggs do not divide at all.

Thus, clinostat rotation, in changing the value of one of the parameters of segregation, takes the process of division beyond the limits of structural stability. Then, individual variations in the structure of the egg may appear in diverse variants of division. Segregation of the cytoplasm continues in individual groups of blastomeres throughout division (in particular, in the end zone of the blastula) and probably for this reason, sensitivity to clinostat rotation is maintained until the beginning of gastrulation.

Thus, after clinostat rotation in various eggs, cells of diverse origins appear in a single region. The cells undergo different numbers of divisions and contain cytoplasms with different compositions. The spectra of elementary morphogenetic movements of which they are capable also differ.

IV.2.7. The Effect of Clinostat Rotation on Gastrulation

Clinostat rotation always produces a spectrum of gastrulation anomalies which is wider the earlier the rotation is begun and the longer it is continued. We observed highly various morphologies in single regions of the embryo while the morphologies

of different regions are related to each other differently: the various embryo structures which were obtained are presented in Fig. 29. Even in instances in which the spatial organization of the embryo was very peculiar, and development proceeded in a manner unusual in amphibians, we noted local similarities in structure to those which occur in normal gastrulation. For example, when the basic mass of cytoplasm practically did not divide, or when only its surface layer divided, the divided cells were organized in a well ordered sheet--the roof of an atypical blastocoel. By the time that the control embryos began gastrulation, characteristic elements of gastrulation movement were observed on the edge of the "blastodisc" which had been formed. The edge of the sheet was turned under and the edge cells receded into the flow (Fig. J).

Whatever the anomalies in the structure of the embryo, regions exist in which it is possible to identify the morphology of /163
of a region of a normal gastrula at some stage of development. However, despite morphological similarities, the conditions of these regions may be completely different from those of a homologous region during normal gastrulation (Holtfreter, 1943; Lovtrup, 1975; Keller, 1975, 1976). Thus, gastral invagination may be shifted to any position within the limits of the vegetative hemisphere of the egg (Fig. 29, A, H), and membranes from a single structure may be formed from material from practically any part of the embryo (Fig. 29, C2, D3, E3).

Gastrulation movement may be completely absent in one part of the edge zone and proceed normally in another: the forward movement of the gastrulation front of invagination may occur on the dorsal side, while the side lips of the blastopore are not developed. In addition, the material from the edge zone may recede inside the embryo at the same time as material from the presumptive entoderm remains completely on the surface (Fig. 29, E3, H2) and, inversely, some of the cells of the vegetative hemisphere are immersed in anomalies inside.(Fig. 29, I). Sometimes no signs of gastrulation are observed externally, however, in such cases, internal cells from the edge zone form structures which are homologous with the blastopore lip, although the surface cells of this zone are not implicated (Fig. 29, C1).

Finally, in various regions of a single embryo, processes may occur which are related to various stages of normal gastrulation: when the top of gastrulation invagination reaches almost to the lower boundary of the blastocoel, the roof of the blastula, although it has multiple layers, remains homogeneous in structure (Fig. 29, A1). Inversely, in the absence of invagination, the roof may be subdivided into sheets which have structures typical of the normal ectoderm at relatively late stages of gastrulation (Fig. 29, G2). Thus, the morphological transformations which encompass separate regions of the embryo flow to a considerable degree automatically so that a single morphology is repeated by it-

self, irrespective of its stage of development or of the environment in which it is found.

Clearly, transformations which preserve their characteristics in various combinations with each other must be individually stable: when they are harmonically combined they are able to generate unlimited variations of stable morphologies in gastrulation.

In fact, we were able to demonstrate that all of the variants of gastrulation which arise after clinostat rotation may represent various spatial-time compositions of single combined elementary morphogenetic processes (Dorfman, Cherdantzev, 1977b). These processes (for the separation of which a special formally fundamental procedure is necessary) are structurally resistant to transformations in tissue morphologies and are propagated through the tissue in a wave which catches increasingly new cells. The shift from one local morphology to another may occur either as an "impact wave" moving in a narrow boundary which separates cells which have been shifted and those which have not been, or as "evolvements", when the transition between local morphologies unfold continuously in space (Fig. 30).

Thus, clinostat rotation which destabilizes the creode of normal segregation is, in this way, a method for separating discrete elements in the complex gastrulation process in ecaudate /167

amphibians.

We recall once again that the destabilization of this creode leads primarily to the phenomenon that at the blastula stage the mutual arrangement of the regions of the embryo which correspond to polyclones of different origins becomes relatively unordered and variable. Nevertheless, qualitatively different anomalies in development arise only after several nodal stages have passed. We must consider that, specifically in these stages (and not at all in intermediate stages), several elements of spatial order in the arrangement of these regions become necessary for their normal determination. Stages of development which are nodal in this sense are inevitably sensitive to local disturbances in the organization of the embryo, and consequently, to external influences which arise from divergences in the rates of morphogenetic processes flowing in various sections of the embryo; thus they must be related to the so-called "critical periods of development" (Svetlov, 1960; Goldschmidt, 1954).

In ecaudate amphibians the first such snode--the stage at which individual variations in morphologies are smoothed and the embryo acquires a more or less standard structure, is that during which the dorsal lip of the blastopore is established. The increase in individual variations in division which result in heterochronism between the ageing potential to various morphogenetic movements may lead to an incomplete regulation of development in

this node and we will observe variations in the begining of gastrulation. In this case, the adjustment may be completed only at the second node--the stage during which the side lips of the blastopore are established, or even at the third node--where locking of the blastopore (late gastrula) begins. Clearly, if development after any one of these nodal stages is highly disorganized, the embryos with sharply anomalous structures will die quickly.

Direct experiments (i.e., those done in space flight conditions) on the effect of zero gravity on the early development of amphibians are sparse. We note in particular one such experiment (Young, Tremor, 1968) in which previously cooled eggs of Rana pipiens were put in conditions of a space flight beginning from the stage of 2-4 blastomeres. The embryos, which were returned to earth in considerably later stages of development, did not exhibit any important morphological anomalies. It is understood that anomalies of development in both embryos which had adjusted any early disorganization and those which had met early deaths were not considered.

IV.3. The Effect of Gravity on the Development of Other Vertebrates /168

Immediately following the publication of Pfluger's first experiments, Rauber attempted to repeat the rotation experiments on the dividing eggs of trout (Rauber, 1884; see Bogdanov, 1888). Using special tweezers he hardened the shell of the fertilized

egg and fixed it in a position so that the blastodisc was underneath. Significant anomalies arose in the course of segmentation. Rauber was the first to conduct experiments on centrifuging eggs (and also the first to work with trout embryos). In this case the course of development was normal, although we must keep in mind that the accelerations used were very low (approximately 1 g).

For a very long time no further work of this type was done on the embryos of bony fish. On the other hand, it is well known that it is unnecessary for the blastoderm of bony fish to be in contact with the yolk during the early stages of its development in order for its morphological differentiation to occur (see Rudnick, 1955; Devillers, 1961; Ignat'eva, 1965; Kostomarova, 1965). The necessary time of contact depends to a certain degree on the relative quantity of egg yolk (Ignat'eva, 1974). Furthermore, at least in Carassius carassius and Fundulus heteroclitus, the significance of various regions of the yolk changes in the course of development, so that in Carassius the lower 2/5 of the yolk may be removed at the two blastomere stage without impairing further development, while at the four blastomere stage this may be done to the entire lower half of the yolk, and finally at the 8 blastomere stage only the part of the yolk which directly adjoins the blastoderm is essential (see Devillers, 1961). In addition, if the egg is centrifuged immediately after fertilization, the blastoderm acquires the ability to self differentiate even at the two blastomere stage. From this, and also on the basis of other data (see

Ignat'eva, 1965; Ignat'eva, Rott, 1970) it follows that the essential factor in morphological differentiation of the blastoderm is the transfer of "morphogenetic matter" from the yolk to the blastoderm (this obviously depends on the effect of gravity).

In one way or another this again provides the basis for posing the question of the effect of gravity on the development of bony fish. Direct experiments were conducted on the embryos of Fundulus heteroclitus to study the effect of zero gravity (Baumgarten et al., 1975; Scheld et al, 1975). However, since in these experiments the embryos were placed in the conditions of a space flight beginning only with the stage at which segmentation was completed (after 32 hours of development), the results were clearly contradictory. This was in full agreement with clinostat experiments conducted on the same species; beginning with the gastrula stage, clinostat rotation had no effect on the course of morphogenesis. However, if clinostat rotation was begun somewhat earlier (from the stage of the middle blastula, we must remark that the embryos were cooled in advance), significant anomalies occurred in the structure of the axial complex. These anomalies were evidently related to the divergences between the rates of various morphogenetic processes which participate in formation of the complex. Before this, discrepancies in the morphogenetic movements of gastrulation occurred without fail (the normal development of these movements was studied by Ballard; Ballard, 1966, 1968; see also Trinkaus, 1972). /169

Data on the effect of gravity on other species of fish reduce to individual remarks on the correlation between the direction of rotation of the egg following fertilization (when it moves to its "standard" orientation in the gravitational field) and the position of the axial complex (Detlaf, Ginzburg, 1954; Claver, 1962).

This type of correlation was studied in detail in the development of birds (Clavert, 1962). Until the appearance of the zona pellucida, all of the directions in the blastoderm are equipotential and only later is one of them selected as the direction of the long axis of the complex. The selection of the direction along which the embryonic stria will be located may be determined by the slope of the blastoderm relative to the vertical. Gastrulation then begins in the uppermost part of the blastoderm. In this manner it is simple to explain the relation between the orientation of the axial complex and the direction in which the egg rotates in the oviduct.

This is evidently also true for reptiles.

From the vast amount of work which has been devoted to the effect of centrifuging on the development of invertebrates we see the surprising resistance of these creatures to its effects, at least in the overwhelming majority of species (Morgan, 1927;

Raven, Beenakkers, 1955; Overton, Raab, 1967, Guerrier, 1968). In fact, the effect of centrifuging begins to be evident only at accelerations on the order of hundreds and even thousands of g. Evidently, in the majority of invertebrates both the polar and bilateral organization of the embryo are maintained not so much by the character of the distribution of the internal components of the egg, as by the stability of its cortical layer.

Therefore, the sparse data (see Parfenov, 1975; Smitt, 1975) obtained from direct experiments on the effect of zero gravity on the development of invertebrates (primarily of the higher insects) shows clearly that if gravity plays some role in development, it is anvery insignificant one. Nevertheless, the final opinion on this matter may only be drawn after additional investigations are conducted.

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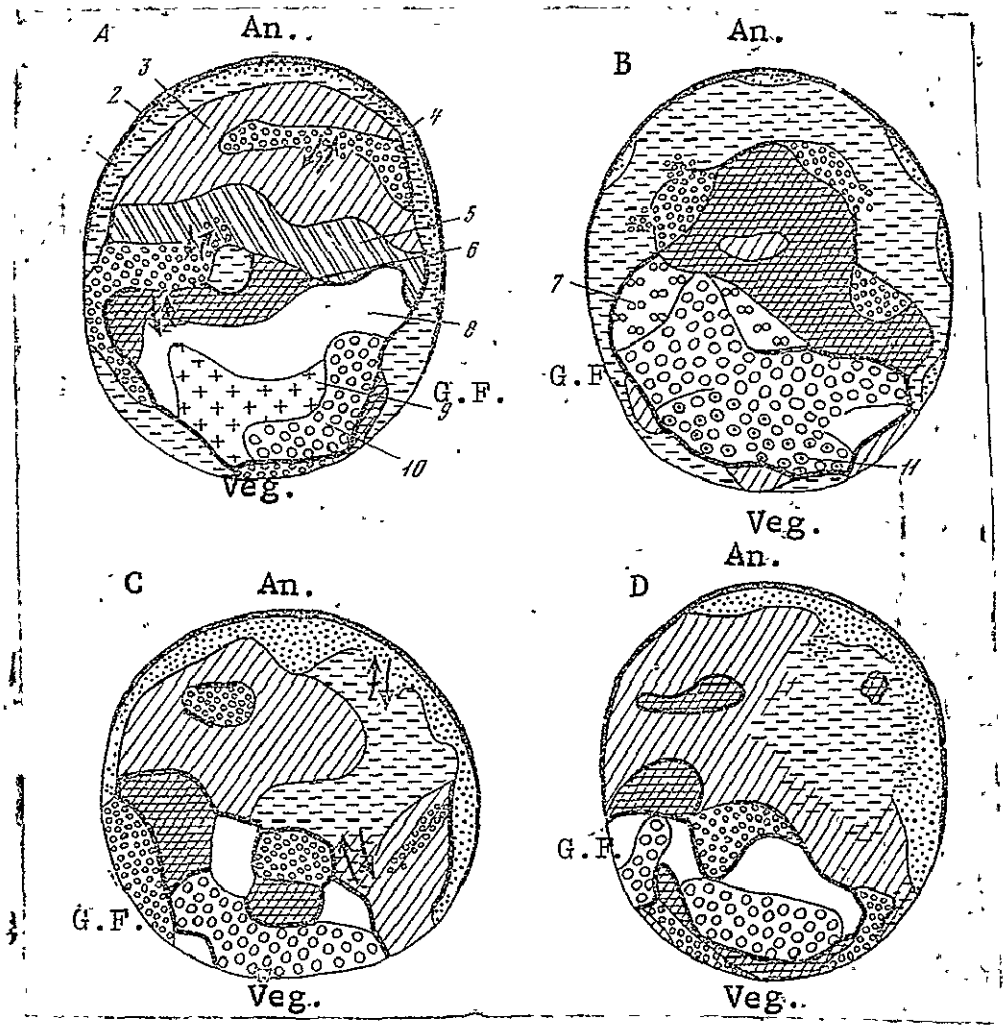


Fig. 26. Plans of the zonal organization of the yolk in the egg of the grass frog (Original Figure done by Ya. G. Dorfman, N. I. Sheinoy, see also Dorfman, Cherdantsev, 1977a).

The distribution of yolk granules of various sizes in normally developed (A,B) and clinostatically rotated (C,D) eggs from a single clutch (10 min before the appearance of segmentation sulci I). Zones which contain granules similar in size which were hatched similarly. An.--animal, Veg.--vegetative pole; G.F. grey falx. Zones (1-II) are numbered according to the increase in the mean size of the yolk granules. The mean size of the granules in zone 1--3 μm, in zone II--7 μm. Thick lines--section of the boundary between zones for which the relation $(d_1-d_2)/(d_1+d_2)<1/10$ holds, where d_1 and d_2 are the mean sizes of the yolk granules in zones directly adjacent to the interfaces. The arrangement of thus separated "narrow" zones is profoundly disturbed by clinostat rotation. Opposing arrows--region of mixing granules from neighboring zones.

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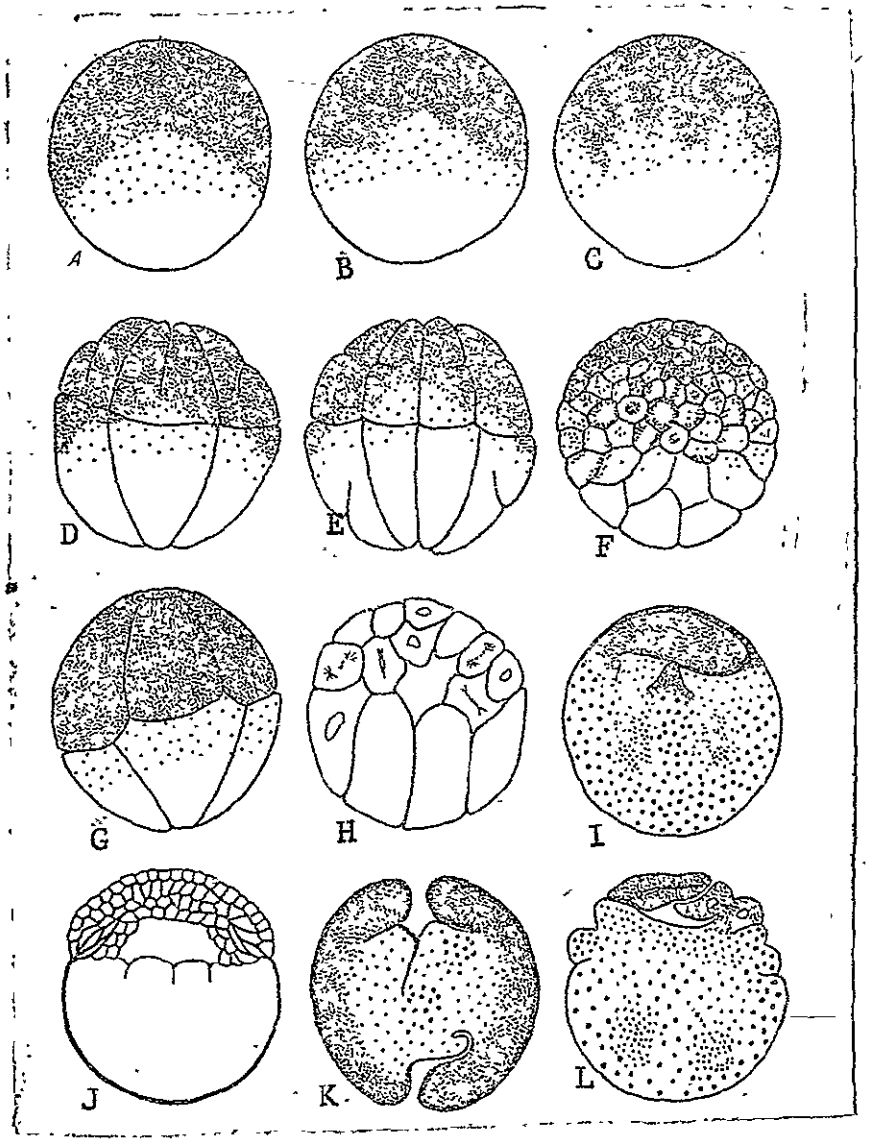


Fig. 27. Some anomalies in segmentation in ecaudate amphibians resulting from clinostat... rotation.

A-G--external views of the eggs, H-L--sections

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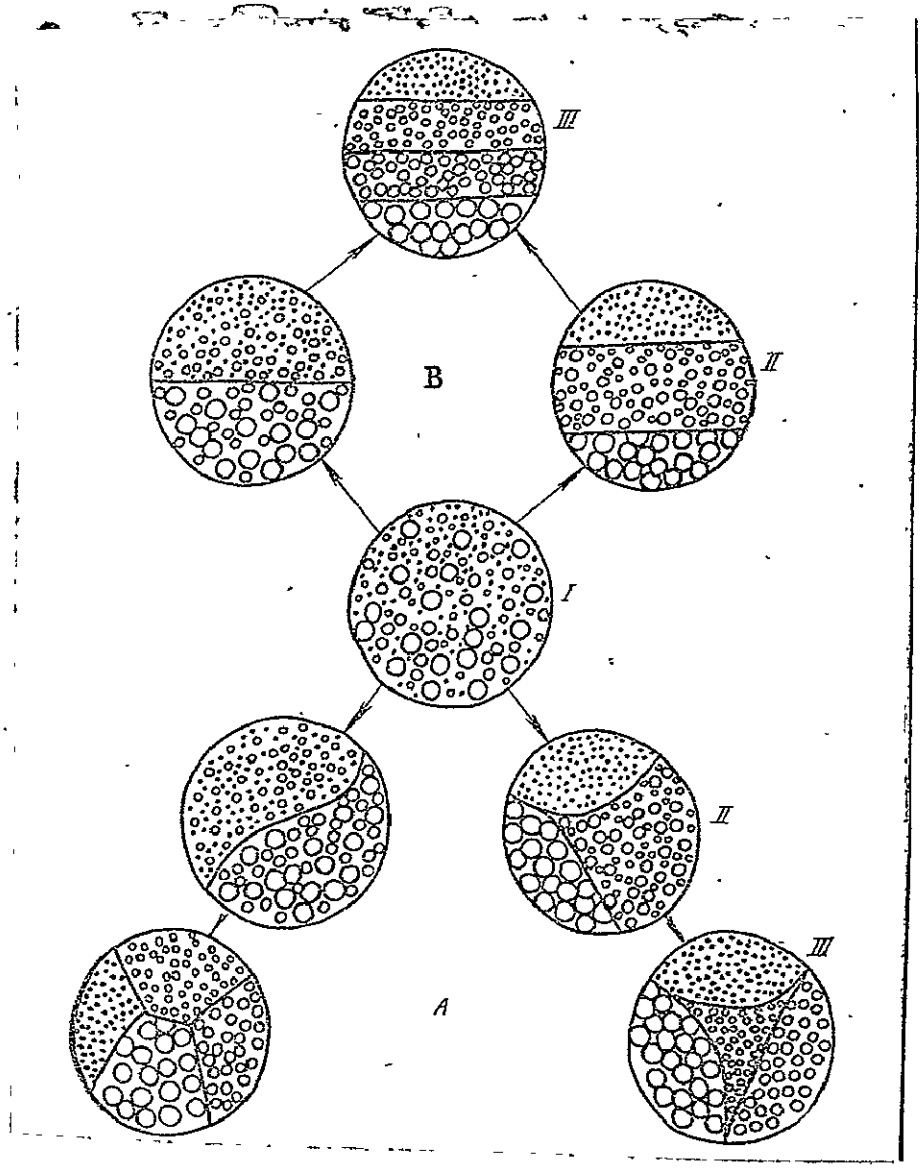


Fig. 28. A scheme explaining the effect of variations in the sequence of separating phases of the yolk which are segregated from each other on its final structure in zero gravity (A) and in a normal gravitational field (B)

I-III--successive stages of segregation. Circles of different sizes designate yolk granules which are differentiated by their maximum diameter (they are broken down conditionally into four classes). The distribution density of the different sized circles reflects the concentration of granules of the corresponding class in a given region of the egg. Gravity creates concentration gradients for each component of the yolk in heterogeneously composed zones and regulates the arrangement of the zones. In the gravitational field, variations in the succession of separation of the phases does not affect the final zonal organization of the egg.

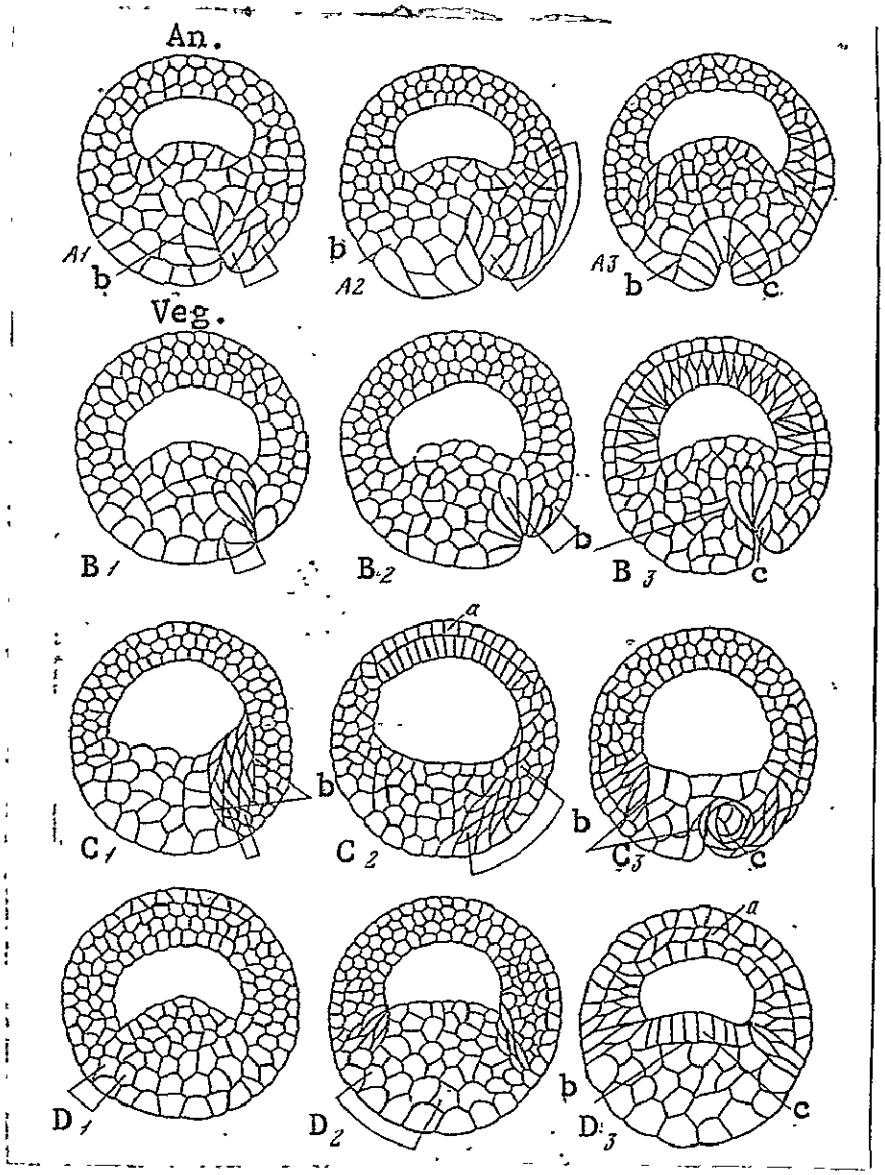


Fig. 29. Several variants of the structure of clinostatically rotated embryos of Rana temporaria in the gastrulation stage (sagittal sections)

An.--animal, Veg.--vegetative pole of the egg, on the right--dorsal side. A1--I1--examples of the flow of corresponding elementary processes in the form of an impact wave, A2--I2--in the form of evolvements, A3-I3--typical combinations of these processes with each other; a--disks with different morphologies may occupy various positions in the embryo; b--immobile boundary relative to the cellular material between regions with various local morphologies which arise as a result of disturbances in normal ooplasmic segregation; c--local morphology which arises due to combinations of the morphogenetic processes. Brackets--the positions of impact waves and evolvements of the corresponding elementary processes.

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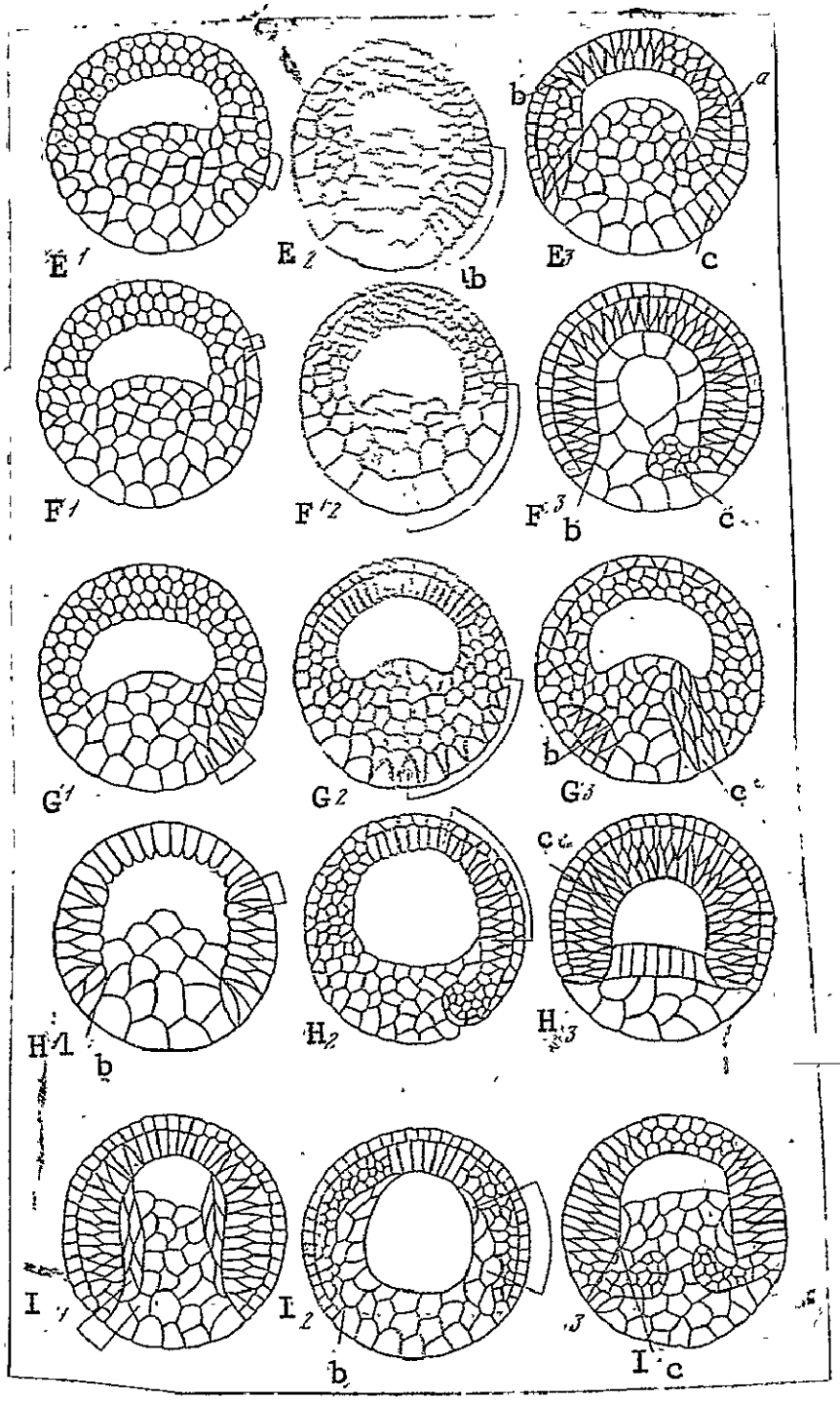


Fig. 29. (end)

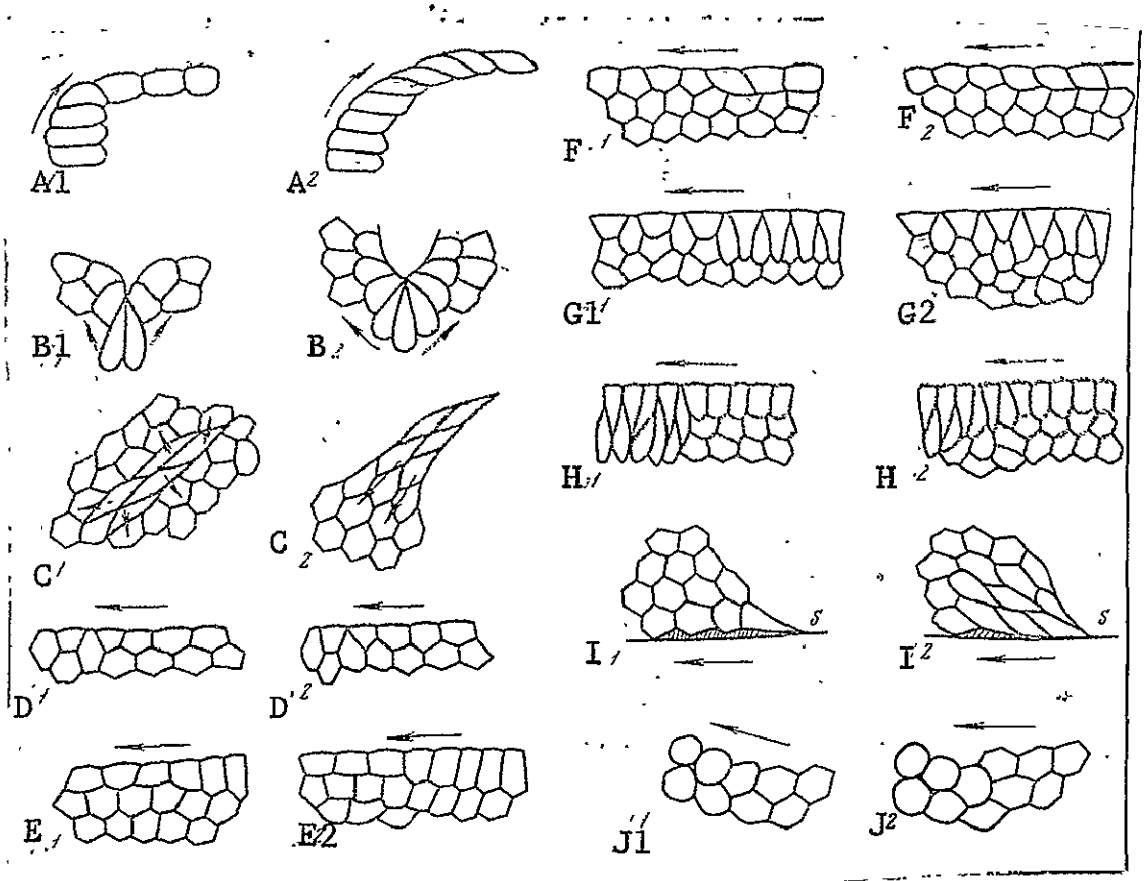


Fig. 30. Elementary morphogenetic processes which comprise gastrulation in ecaudate amphibians

Each elementary processes flows either in the form of an impact wave (1) or of an evolvement (2). The arrows indicate the direction in which the cells, which have already changed their forms, induce similar changes in the forms of other cells. The reverse is destructive and flows autonomically in each section.

A--formation of flows in the membrane, or "inversion" of the membrane";
B--formation of a dome from the ampullaceous cells;
C--formation of flows in the polygonal cell layer;
D--division of the membrane;
E--ordering of the membrane;
F--formation of an integrating surface;
G--immersion of the cells in the layer;
H--secondary ordering of the individually polarized cell membrane;
I--formation of a flow along the external substrate (S);
J--establishment of general adherence in the layer

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